RESEARCH ARTICLE

Shifts in microbial community structure and function in stream sediments during experimentally simulated riparian succession

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Abstract

Successional changes of terrestrial vegetation can profoundly influence stream ecosystem structure and function. We hypothesized that microbial enzyme production and community structure in stream beds depend on terrestrial litter inputs that reflect different stages of riparian succession. Outdoor experimental channels were supplied with leaf-litter of varying quantities and qualities to mimic litter supply during five successional stages: (1) an initial biofilm stage; (2) an open-land stage with grass litter; (3) a transitional stage with mixed grass and birch litter; (4) an early forest stage with birch litter; and (5) an advanced forest stage with 2.5 × the amount of birch litter. Mean potential activities of nitrogen- and phosphorus-acquiring enzymes in sediments (20.7 and 67.3 μmol g^{-1} dry mass) were 12–70 times greater than those of carbon-acquiring enzymes (0.96–1.71 μmol g^{-1} dry mass), with the former reduced 1.3–8.3-fold in channels with tree litter. These patterns could suggest gradually diminishing nutrient limitation of microbial activity during riparian succession, potentially linked both to an increasing supply by the added litter and to a lower nutrient demand as algal biomass and labile carbon supply by photosynthetic exudates declined. As the observed shifts in nutrient-acquiring enzymes were reflected in changes of sediment microbial communities, these results indicate that both the type and density of terrestrial vegetation control microbial community structure and function in stream sediments, particularly enzyme production related to nutrient cycling.

Introduction

Concepts of ecosystem succession in recently created landscapes such as volcanic lava fields (Vitousek, 2004), forefields of receding glaciers (Milner *et al.*, 2011) or post-mining areas (Hüttl & Weber, 2001) emphasize a sequence of successional stages shaped largely by the development of terrestrial vegetation (Walker & del Moral, 2003). Streams forming in these landscapes are intimately linked to their terrestrial surroundings (Webster, 2007), implying that their metabolism and communities vary profoundly along the successional path from bare land to mature forests. In open-land streams where riparian vegetation is absent or restricted to herbs, metabolism is initially dominated by biofilm processes. This includes instream primary production by benthic algae (Bernot *et al.*, 2010) fuelling heterotrophic biofilm communities (Jones *et al.*, 1995; Romaní & Sabater, 1999), in addition to mobilized ancient organic matter stores (Bardgett *et al.*, 2007) and aerial inputs (Hodkinson *et al.*, 2002). Later, as herbal vegetation encroaches, plants near and within streams provide increasing amounts of terrestrial plant litter, while sunlight still reaches the stream bottom and enables continued algal growth (Huryn *et al.*, 2001; Acuña *et al.*, 2010). Eventually, however, shrubs and trees form a closed canopy above stream channels up to a few metres wide, limiting light penetration and algal development (Sabater *et al.*, 2000) while delivering large amounts of plant litter (Benfield, 1997). In addition to these quantitative

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changes, the quality of plant litter shifts along the successional trajectory in terms of elemental stoichiometry and concentrations of recalcitrant or inhibitory constituents such as lignin and polyphenolics, especially when graminoids and forbs are replaced by shrubs and trees (Yuan & Chen, 2009).

How will microbial communities and activities in stream sediments respond to changing litter inputs as landscapes and riparian vegetation undergo successions? Will changes be idiosyncratic or smooth, reflecting the gradual changes in resource supply occurring during the successional trajectory? How important is the quantity and quality of litter in determining microbial community composition and activities? Such questions can be addressed by analyzing microbial communities and activities along chronosequences, for example in glacier forefields (Brankatschk *et al.*, 2010; Milner *et al.*, 2011) or on oceanic volcanic archipelagos (Vitousek, 2004). However, as many factors vary simultaneously during succession, the chronosequence approach has limited power to pinpoint the significance of a particular factor such as litter supply. An alternative approach is to conduct manipulative experiments in model systems, where all factors other than the one of interest are kept constant. Such model systems are necessarily simplified but bear the important advantage of precluding influences of any known or unknown confounding factors.

Given the importance of organic matter quality and quantity in determining microbial community composition (Comte & del Giorgio, 2009), abundance (Fischer *et al.*, 2002) and activity (Sobczak & Findlay, 2002), structural and functional aspects of microbial communities in stream sediments are expected to vary with changing litter supply as riparian succession proceeds. One way to assess microbial activities is to determine potential extracellular enzymes activities, an approach that can yield more detailed information than bulk activity measurements such as respiration, including insights into biogeochemical processes other than those involved in the carbon cycle (Sinsabaugh *et al.*, 2008). What is more, ratios of potential extracellular enzyme activities have been suggested to serve as indicators of the relative importance of different biogeochemical processes (Sinsabaugh *et al.*, 2009; Sinsabaugh & Follstad Shah, 2010), which tend to be driven by different microbial communities (Peter *et al.*, 2011; Teeling *et al.*, 2012). Accordingly, potential extracellular enzyme activities in stream sediments were found to vary with the quantity and quality of the available organic matter (Findlay *et al.*, 2003; Romaní *et al.*, 2004) and were often accompanied by differences in abundances (Artigas *et al.*, 2009) and shifts in microbial community composition (Findlay *et al.*, 2003; Docherty *et al.*, 2006).

The experiment presented here aimed at assessing the responses of microbial community structure, abundance and activities to a key component of landscape and stream succession: the development of riparian vegetation leading to increasing amounts and changing quality of plant litter and concomitant increases in shading. We hypothesized that microbial community structure in stream sediments and capacities of microbial carbon, nitrogen- and phosphorus-acquiring enzymes involved in organic matter degradation change either gradually or abruptly along a litter-supply gradient, reflecting different successional stages. Specifically, we hypothesized that microbial communities and enzyme patterns are influenced by (1) the input of any type of leaf litter; (2) the presence specifically of leaf litter from trees; (3) litter quality (grass vs. tree litter); (4) litter quantity; and (5) litter mixing (presence of both grass and tree litter).

Materials and methods

Experimental design

We conducted an experiment in 15 outdoor channels simulating sand-bed streams receiving allochthonous litter inputs at five stages of simulated riparian succession: (1) an initial biofilm stage; (2) an open-land stage characterized by graminoids and forbs; (3) a transitional open-land and forested stage; (4) an early forest stage; and (5) an advanced forest stage. The straight experimental channels ($4.0 \times 0.12 \times 0.12$ m) were placed side by side on tripods 1 m tall (Supporting Information, Fig. S1). They were set up in the field next to a recently created experimental catchment (Chicken Creek; 51°36′N, 14°16′E) in eastern Germany (Gerwin *et al.*, 2009; Gerull *et al.*, 2011). This ensured natural light, temperature and other environmental conditions. All channels received full sunlight. However, the sediments in channels simulating advanced successional stages were partly (stages 2 and 3) or fully (stages 4 and 5) shaded by a surface layer of leaf litter. Water was partially recirculated through a pipe connecting the upstream and downstream ends of each channel. The channels were filled with a 4-cm layer of sand collected from a dry stream reach in the catchment and supplied with well oxygenated stream water from the catchment, which was collected in a subterranean tank before being delivered to the experimental channels (Frossard *et al.*, 2012a). Water flow was maintained by a pump at 1.7 ± 0.3 cm s^{-1} (mean $\pm$ SD). Sediments were continuously submerged, although water depth was shallow (1 cm). Dissolved organic carbon (DOC) was abundant, averaging 9.8 mg L^{-1}, but hardly bioavailable (Gerull *et al.*, 2012). Other physico-chemical characteristics of the water in the channels are summarized in Table 1.

Table 1. Means and standard deviations of sediment organic matter (OM) and physico-chemical parameters of water in 15 experimental stream channels grouped in five different successional stages and sampled on four occasions

Incubation time	Stages	NO_2^- (µg L^{-1})	NO_3^- (µg L^{-1})	NH_4^+ (µg L^{-1})	PO_4^{3-} (µg L^{-1})	SO_4^{2-} (mg L^{-1})	DOC (mg L^{-1})	OM (mg g^{-1})	Temperature (°C)	pH	Specific conductivity (µS cm^{-1})
1 day	1	< 1	45.6 ± 5.7	11.9 ± 6.5	4.4 ± 1.6	368 ± 3	14.4 ± 1.0	0.92 ± 0.08	20.9 ± 0.6	7.62 ± 0.09	859 ± 1
	2	1.1 ± 0.1	66.0 ± 9.6	14.4 ± 11.5	5.2 ± 0.7	371 ± 10	17.4 ± 1.2	0.89 ± 0.04	23.3 ± 3.0	7.71 ± 0.02	886 ± 16
	3	1.0 ± 0.02	71.6 ± 17.9	14.9 ± 11.5	4.5 ± 1.1	370 ± 7	20.6 ± 0.8	0.87 ± 0.17	23.5 ± 2.9	7.71 ± 0.02	900 ± 6
	4	1.4 ± 0.01	73.2 ± 10.1	40.4 ± 0.04	5.6 ± 1.5	361 ± 19	24.1 ± 2.8	0.84 ± 0.01	27.1 ± 1.6	7.52 ± 0.16	902 ± 31
	5	2.3 ± 0.3	163.6 ± 2.8	10.3 ± 4.5	20.4 ± 12.1	360 ± 29	38.0 ± 0.9	0.91 ± 0.06	27.3 ± 0.4	7.72 ± 0.03	897 ± 33
6 weeks	1	< 1	30.6 ± 3.9	12.9 ± 0.03	1.5 ± 0.4	358 ± 17	12.5 ± 0.5	0.96 ± 0.09	5.3 ± 0.1	7.38 ± 0.02	905 ± 22
	2	< 1	41.1 ± 6.3	5.4 ± 0.01	1.5 ± 0.1	371 ± 9	12.5 ± 1.8	0.81 ± 0.04	5.6 ± 0.2	7.39 ± 0.04	917 ± 5
	3	< 1	35.8 ± 3.9	16.3 ± 14.6	2.1 ± 0.9	350 ± 14	12.7 ± 1.2	0.76 ± 0.04	7.1 ± 1.6	7.41 ± 0.02	924 ± 24
	4	< 1	39.7 ± 2.5	40.1 ± 0.04	2.3 ± 0.9	364 ± 9	12.7 ± 1.2	0.73 ± 0.02	9.2 ± 0.4	7.46 ± 0.02	913 ± 16
	5	< 1	41.7 ± 5.8	13.0 ± 9.8	2.4 ± 0.2	361 ± 10	11.9 ± 1.7	0.73 ± 0.05	11.0 ± 0.4	7.48 ± 0.07	909 ± 15
8 weeks	1	10.1 ± 1.3	65.0 ± 14.8	24.3 ± 8.6	1.9 ± 0.2	200 ± 8	8.4 ± 0.2	1.08 ± 0.06	4.6 ± 0.1	7.39 ± 0.13	553 ± 14
	2	10.3 ± 3.2	54.3 ± 17.1	12.5 ± 7.6	1.9 ± 0.6	215 ± 14	8.1 ± 0.4	0.99 ± 0.07	4.6 ± 0.1	7.45 ± 0.08	601 ± 24
	3	12.6 ± 0.7	52.8 ± 10.3	11.6 ± 3.7	2.3 ± 0.2	188 ± 52	7.4 ± 1.7	0.96 ± 0.03	4.8 ± 0.3	7.37 ± 0.23	538 ± 117
	4	6.6 ± 4.6	54.5 ± 11.6	18.1 ± 7.2	1.9 ± 0.4	210 ± 25	8.0 ± 1.2	0.91 ± 0.16	5.1 ± 0.04	7.46 ± 0.10	588 ± 45
	5	6.3 ± 4.5	44.3 ± 1.6	15.4 ± 11.5	2.4 ± 0.2	221 ± 13	8.2 ± 0.4	1.12 ± 0.42	5.2 ± 0.1	7.49 ± 0.05	628 ± 26
10 weeks	1	2.6 ± 0.6	39.6 ± 5.8	9.1 ± 0.5	1.7 ± 0.4	265 ± 8	7.9 ± 0.3	0.92 ± 0.09	9.9 ± 0.1	7.30 ± 0.03	745 ± 9
	2	2.4 ± 0.8	43.0 ± 13.8	10.5 ± 2.5	1.9 ± 0.6	268 ± 5	8.2 ± 0.2	0.91 ± 0.08	9.7 ± 0.1	7.31 ± 0.03	758 ± 5
	3	2.3 ± 0.9	33.9 ± 3.3	11.4 ± 5.8	2.0 ± 0.7	261 ± 11	8.2 ± 0.4	0.83 ± 0.14	10.1 ± 0.5	7.45 ± 0.08	766 ± 3
	4	1.9 ± 0.9	41.3 ± 9.7	8.3 ± 1.3	2.1 ± 0.7	257 ± 3	8.0 ± 0.3	0.76 ± 0.17	10.7 ± 0.05	7.40 ± 0.01	771 ± 7
	5	1.1 ± 0.1	41.1 ± 4.2	9.8 ± 1.1	2.1 ± 0.5	258 ± 7	8.0 ± 0.2	0.74 ± 0.16	10.6 ± 0.1	7.40 ± 0.07	770 ± 5

DOC, dissolved organic carbon.

To simulate shifts in plant litter inputs into streams during the five stages of stream succession, the experimental channels were stocked with grass or tree litter. Wood small-reed [*Calamagrostis epigejos* (L.) Roth] was chosen as grass because it was dominant during the early successional stages in the experimental catchment. Silver birch *Betula pendula* (L.) Roth was chosen because it is a common tree species on sandy soils in the region, where it can form extensive stands. Both types of litter were collected from single stands in autumn 2007. They differed greatly in texture, and tree litter also had higher concentrations of nitrogen and especially phosphorus (6.9 mg N g^{-1} dry mass, 1.2 mg P g^{-1} dry mass) than the grass litter of wood small-reed (3.4 mg N g^{-1} dry mass, 0.16 mg P g^{-1} dry mass), which produces low-nutrient litter compared with other grassland species (Yuan & Chen, 2009). Experimental treatments included (1) no litter addition (i.e. bare stream bed); (2) 100 g m^{-2} of grass litter (*c.* 20% of the stream bed covered); (3) 50 g m^{-2} of grass litter and 50 g m^{-2} of tree litter (*c.* 60% cover); (4) 100 g m^{-2} of tree litter (100% cover, single layer of leaves); and (5) 250 g m^{-2} of tree litter (100% cover, two to three leaf layers). The five treatments were replicated three times, resulting in a total of 15 experimental channels. One fifth of the litter was mixed into the sediment when filling the channels to mimic the natural burial of litter in sandy sediments. The rest was distributed on the sediment surface.

Stream water suspensions containing natural microbial assemblages were poured in all channels as natural inoculum to boost microbial colonization of the channels. To generate the inoculum, 24 g dry mass of mixed grass and tree litter (various local species) was collected in three local open-land and forest streams. To detach bacterial cells from the leaf surfaces, half of the litter was sonified in an ultrasonic bath (3 min, 35 W output; Buesing & Gessner, 2002) containing 8 L of Chicken Creek water in the experimental catchment. The other half was aerated for 48 h in 8 L of Chicken Creek water to induce fungal sporulation. The 15 channels each received 500 mL of the two suspensions. The purpose of this inoculum derived from natural streams in both early and late successional stages was, in accordance with Baas Becking's hypothesis (de Wit & Bouvier, 2006), to provide 'everything' from which the environment could select. After inoculation, water was completely recirculated in individual channels for 24 h to facilitate establishment of the added microorganisms. Subsequently, the channels were supplied with stream water from the catchment such that 5% of the water volume in the channels was replaced during each cycle. This resulted in an average water renewal time of 6 h.

Sampling and water chemical analyses

Water and sediment samples were collected after 6, 8 and 10 weeks. A syringe (2.8 cm inner diameter, lower end cut off) was used to take three sediment cores per channel to a depth of 4 cm (i.e. over the entire sediment depth). We covered the lower end of the syringe with a small plastic card when retrieving the syringe from the sediment to avoid loss of sediment material. The cores were taken at random spots in each channel, determined independently of whether the sediment was covered by leaf litter. Any surface or buried litter in sediment cores was discarded. The three cores were pooled to obtain a single representative sample per channel and stored frozen (−20 °C) for 3 months. Additional sediment cores were collected to a depth of 1 cm for chlorophyll *a* analyses. Surface water was collected with a syringe, immediately filtered through pre-washed 0.45-μm pore size cellulose acetate filters, frozen at −20 °C and later analyzed for DOC and nutrients (NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}, SO_4^{2-}). Conductivity, pH and temperature were measured directly in the channels with handheld WTW probes (Weilheim, Germany). Sediment organic matter content was determined as the difference in mass of dried (105 °C) and combusted (4 h at 450 °C) samples.

Potential extracellular enzyme activities

Potential activities of seven extracellular enzymes were assessed on thawed sediment samples using substrate analogues linked to one of two fluorescent molecules (4-methylumbelliferone, MUB; or 7-amino-4-methylcoumarin, AMC) or to 3,4-dihydroxyphenylalanine (L-DOPA) (Frossard *et al.*, 2012b). Although freezing has been observed to affect potential extracellular enzyme activities (Chróst & Velimirov, 1991; DeForest, 2009), effects on the patterns of individual enzymes are likely to be small in our study because channels were filled with the same sandy substrate and other environmental conditions were also similar across treatments. The seven enzymes measured were alkaline phosphatase (AP; indicates P acquisition), leucine aminopeptidase (LAP; indicates N acquisition), β-glucosidase (BG; indicates cellulose degradation), β-xylosidase (BX; indicates hemicellulose degradation), β-N-acetylglucosaminidase (NAG; indicates N and C acquisition), phenol oxidase (PO; indicates oxidation of polyphenols such as lignin) and phenol peroxidase (PP; indicates polyphenol oxidation). Assays involved placing 2 g of sediment in 60 mL of 0.1 mM tri(hydroxymethyl)aminomethane buffer (pH 7.5, autoclaved), stirring the slurry, and adding 200 μL to 96-well microplates. The substrate analogues were added at saturation level (50 μL of 200 μM stock solutions = 40 μM final concentration), which was invariably reached in preliminary tests on selected samples for all

enzymes. Fluorescence or absorbance was measured after incubation at 10 °C for 1.5 h (NAG and AP) or 4 h (BG, BX, LAP, PO and PP). NaOH (0.5 N, 10 μL) was added before shaking the microplates and measuring fluorescence on a microplate reader (Tecan Infinite® 200, Männedorf, Switzerland) at an emission wavelength of 445 and 450 nm, respectively, and an excitation wavelength of 365 nm for both types of substrate. Absorbance in the PP and PO assays was measured at 460 nm. Background fluorescence or absorbance from the sediment and substrate analogues was subtracted by measuring sample and substrate controls. Quenching was assessed with a quench coefficient (0.65–1.00), calculated as the quotient of fluorescence of each individual sample spiked with a fluorescence standard (MUB or AMC) and the same standard added to buffer only.

Bacterial and algal biomass

The abundance of bacteria (i.e. *Bacteria* and *Archaea*) was determined by flow cytometry as described in Frossard *et al.* (2012b). Bacterial cells were detached from the sediment with an ultrasonic probe (Buesing & Gessner, 2002), separated from other particles by collecting them in Histodenz® solution (Caracciolo *et al.*, 2005), staining with SYBRGreen I, and counting on a CyFlow® space Flow Cytometer System (Partec, Görlitz, Germany) equipped with a 200-mW solid-state laser (light emission at 488 nm) and volumetric counting hardware (Hammes & Egli, 2005). A conversion factor of 58 fg per cell was used to calculate bacterial biomass from abundance data (A. Frossard *et al.* unpublished data). This factor has been obtained by determining the biovolume of a total of 13 000 bacterial cells from sediment samples taken in 12 different streams (A. Frossard *et al.* unpublished data) and converted to biomass using the biovolume–biomass relationship established by Loferer-Krößbacher *et al.* (1998).

Chlorophyll *a* was used as a proxy of algal biomass. It was analysed spectrophotometrically after extraction with 90% ethanol for 4 min in the dark at 70 °C (Gerull *et al.*, 2011). A conversion factor of 60 mg C mg^{-1} chlorophyll *a* was used (Romaní & Sabater, 2000).

Microbial community structure

The structure of microbial communities was assessed by automated ribosomal intergenic spacer analysis (ARISA). DNA was extracted from frozen (−80 °C) sediment following Frossard *et al.* (2012b). The extraction involved mechanical cell breakage, enzymatic digestion of unwanted cell constituents, and DNA purification. The purified DNA was stored at −20 °C. PCR amplification of the intergenic region of bacterial rDNA was carried out using labelled forward primer 1406F-FAM (5′FAM-TGY ACA CAC CGC CCG T-3′, T=T, C) and reverse primer 23Sr (5′-GGG TTB CCC CAT TCR G-3′, B=G, T, C and R=G, A; Microsynth, Balgach, Switzerland; Yannarell *et al.*, 2003). Although designed to amplify bacterial rDNA, these primers can also bind to chloroplast and mitochondrial DNA, such that the resulting community fingerprints could include algae as well as other eukaryotes. Therefore, we refer to microbial community structure when interpreting ARISA profiles. The mix contained 1 × GoTaq® Flexi reaction buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 mM of each primer, 0.25 mg mL^{-1} bovine serum albumin (BSA), 0.05 U μL^{-1} GoTaq® Flexi DNA Polymerase, and 1 μL of DNA extract. The initial denaturation step (94 °C for 2 min) was followed by 30 amplification cycles consisting of a denaturation step at 94 °C for 35 s, primer annealing at 55 °C for 45 s and an extension phase at 72 °C for 2 min. The final extension was completed at 72 °C for 2 min.

Size and yield of the PCR products was checked on a 2% agarose gel containing a 100-bp ladder (Promega, Dübendorf, Switzerland). A 1-μL volume of PCR product was mixed with 9 μL of HiDi formamide and 0.5 μL of standard LIZ1200 (Applied Biosystems, Rotkreuz, Switzerland) before exposing it to 95 °C for 3 min and subsequent cooling on ice. The intergenic spacer fragments were separated on a 3130XL Capillary Genetic Analyzer (Applied Biosystems) using a 50-cm capillary and a standard GENEMAPPER protocol (Applied Biosystems). The peak pattern was analyzed with PEAK SCANNER V1.0 (Applied Biosystems). Fragments ranging in length from 200 to 1200 bp were analyzed in terms of both relative peak area and simple presence or absence. Profiles of different samples were compared using the script *interactive_binner.r* (Ramette, 2009) implemented in the statistical software R (R Development Core Team, 2011).

Data analysis

Linear mixed-effects models were fitted with the function *lme* from the package *nlme* (Pinheiro *et al.*, 2011) for the statistical software R (R Development Core Team, 2011) to test for differences among successional stages (i.e. litter treatments) at different sampling dates. Response variables included water chemical parameters, potential extracellular enzyme activities, and bacterial and algal biomass. Successional stage and time were treated as fixed factors, with the time variable centred on the 10-week sampling date so that the estimated intercepts represent the situation at the end of the experiment, when the largest effects were expected. A random intercept was included for replicates to account for repeated measures on the same channels. QQ-plots and frequency histograms indicated that residuals did not meet assumptions required for

parametric tests. Therefore, variables (x) were transformed according to $\ln(x + 1)$. Assumptions were met after the transformations. To test our five specific hypotheses, we calculated planned contrasts comparing selected combinations of the five litter treatments corresponding to different successional stages: treatment 1 vs. treatments 2 + 3 + 4 + 5 (Contrast A) tested for the input of any type of leaf litter (Hypothesis A), 1 + 2 vs. 3 + 4 + 5 (Contrast B) tested for the specific presence of leaf litter from riparian trees (Hypothesis B), 2 vs. 4 (Contrast C) tested for litter quality effects (grass vs. tree litter; Hypothesis C), 4 vs. 5 (Contrast D) tested for the effect of litter quantity (Hypothesis D), and 3 vs. 2 + 4 (Contrast E) tested for litter mixing effects (Hypothesis E). In addition, we performed regression analyses with successional stage (i.e. litter treatment) as the independent variable using PASW STATISTICS 18.0. Significance of the deviation of slopes of potential activity ratios (i.e. C-acquiring and lignin-degrading to N- or P-acquiring enzymes) from the 1 : 1 line was tested by calculating for each independent variable the deviations between the observed values and those on the 1 : 1 line and testing by regression analysis whether the slope of the resulting relationship between the independent variable and the deviation was significantly greater than zero.

Non-metric multidimensional scaling (NMDS) analyses were performed with the function *meta.mds* of the package *vegan* implemented in R (R Development Core Team, 2011), based on either the microbial community matrix (relative abundance of each OTU detected by ARISA) or a matrix comprising the seven potential extracellular enzyme activities. Calculations were based on Bray–Curtis distances and 1000 permutations. Permutational multivariate analysis of variance (PERMANOVA) with simulated successional stage (i.e. litter treatment) and sampling date as factors was performed with the function *adonis* in R. Significance of Bray–Curtis distances among centroids of treatment clusters within each community sampled at the same date was assessed among all treatment and also for the specific contrasts described above. Environmental factors were fitted onto the ordinations using the function *envfit* of the R package *vegan*. Significance of the associations was determined by 1000 random permutations.

Results

Physicochemical parameters

DOC, NO_3^-, NH_4^+ and PO_4^{3-} concentrations were significantly higher directly after the start of the experiment (i.e. 1 day after inoculation) than 6–10 weeks later (Table 1). However, none of the chemical parameters varied consistently among channels used to simulate different successional stages. Organic matter in sediment did not vary either among sampling dates (repeated-measures ANOVA, $F_{4,10} = 1.36$, $P = 0.32$) or among channels receiving different litter types ($F_{1,25} = 0.35$, $P = 0.56$). The largest differences among channels were observed for temperature (Table 1), although these were much smaller than the total temperature range experience by the channels during the experiment.

Potential extracellular enzyme activities

AP showed the highest potential activity among the potential extracellular enzymes tested. Average rates exceeded 150 μmol MUB g^{-1} sediment ash-free dry mass (AFDM) h^{-1} in channels without leaf litter, whereas rates in the channels stocked with litter were three times lower (Fig. 1a). LAP, which was used as an indicator of N acquisition, showed the second highest potential activity, ranging from 5.5 to nearly 50 μmol AMC g^{-1} AFDM h^{-1} (Fig. 1b). Potential activities of NAG were lower, with values never exceeding 2.9 μmol MUB g^{-1} AFDM h^{-1} (Fig. 1c). Similarly, the carbon-acquiring enzymes such as BG and BX had potential activities ranging from 0.1 to 1.8 μmol MUB g^{-1} AFDM h^{-1} (Fig. 1d and e). Finally, the potential activity of lignin-degrading enzymes ranged from 1.3 to 1.7 μmol L-DOPA g^{-1} AFDM h^{-1} for PO (Fig. 1f) and from 1.5 to 2.4 μmol L-DOPA g^{-1} AFDM h^{-1} for PP (Fig. 1g).

None of the extracellular enzymes tested differed significantly among the three sampling dates. However, potential activities of AP and LAP significantly varied among the five successional stages ($F_{4,10} = 6.21$ and 11.6, $P < 0.001$), decreasing exponentially ($r^2 = 0.41$ and 0.44, respectively; $n = 45$) along the experimental gradient from channels receiving no litter to those receiving 2.5 times the standard amount of tree litter (Fig. 1a and b). Both enzymes had higher potential activities in the simulated open-land stages (no litter or grass litter added) compared with the channels receiving tree litter (Contrast B: $P = 0.032$ and $P < 0.001$, respectively), but only LAP was higher in the bare channels without litter compared with all others (Contrast A: $P = 0.006$). Likewise, Contrasts C and D showed that LAP was the only enzyme reflecting distinct differences in the quality ($P = 0.005$) and quantity ($P = 0.009$) of added litter. Potential activities of C-acquiring enzymes (BG and BX as well as NAG) and lignin-degrading enzymes (PO and PP) did not differ significantly among treatments, nor were significant interactions observed between successional stage (i.e. litter treatment) and time. The simultaneous presence of litter differing in quality (i.e. mixed grass and tree litter) in a given channel (stage 3) had no significant effect on any of the potential extracellular enzyme activities in sediments (Contrast E).

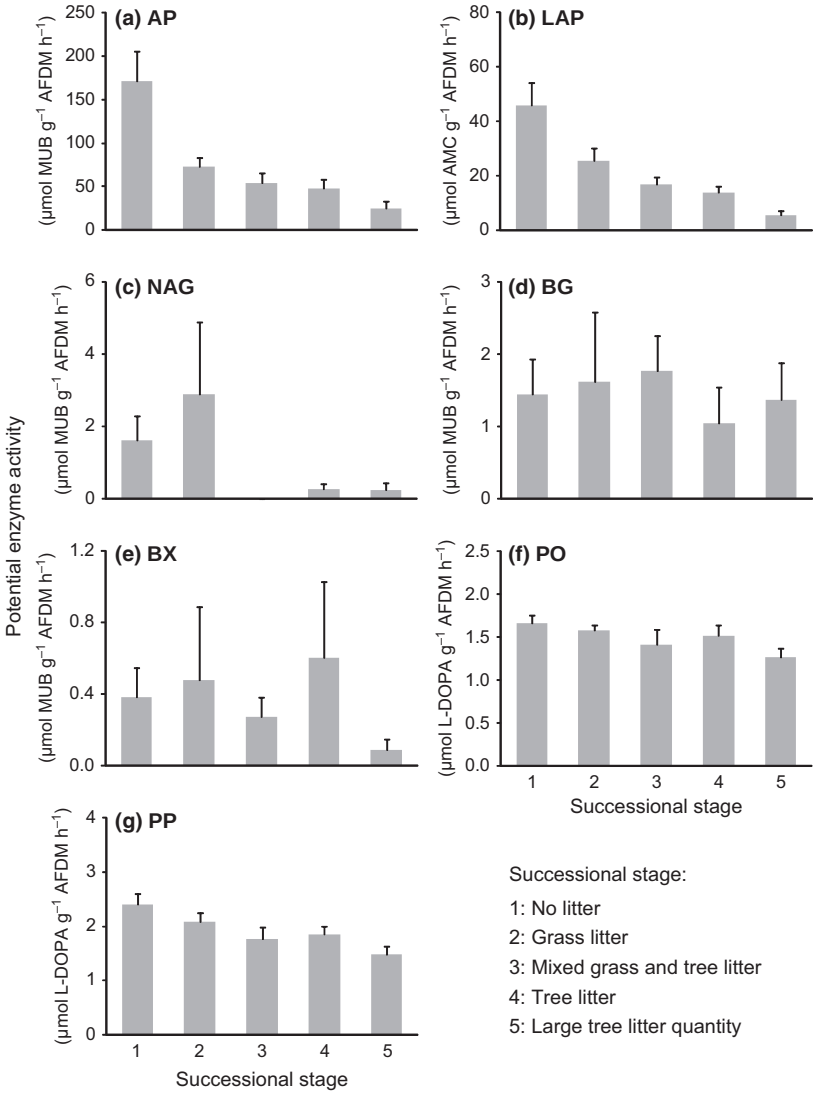

Fig. 1. Potential activities of seven selected extracellular enzymes in the sediment of experimental stream channels supplied with different types and amounts of leaf litter: 1) no litter, 2) grass litter, 3) mixed grass and tree litter, 4) tree litter, 5) large quantity of tree litter. Vertical bars denote standard errors calculated across stream channels (n = 3) based on values aggregated within each channel across sampling dates.

Ratios of log values of potential activities for C-acquiring hydrolytic enzymes (BG and BX) to N- and P-acquiring enzymes ranged from 1 : 1.2 to 1 : 2.1 and from 1 : 1.6 to 1 : 2.3, respectively (Fig. 2a and b), but trends along the litter-supply gradient were not significant. Ratios of the two lignin-degrading enzymes (PO and PP) to N- and P-acquiring enzymes were similar (Fig. 2c and d), ranging from 1 : 1.1 to 1 : 1.3 and from 1 : 1.2 to 1 : 1.4, respectively. However, they increased significantly along the litter-supply gradient (r^2 = 0.30 and 0.22, P = 0.001 and 0.002), mainly due to the observed decreases in potential activities of the N- and P-acquiring enzymes, respectively. Slopes of regression lines of log values of C-acquiring or lignin-degrading to nutrient-acquiring enzyme potential activities ranged from 0.07 to 0.33 and all deviated significantly from the 1 : 1 line ($P < 0.001$).

Fingerprints of potential extracellular enzyme activities in sediment from stream channels stocked with different types and amounts of leaf litter did not form clearly separated clusters in an NMDS ordination (Fig. 3). However, samples were broadly arranged along the litter-supply gradient from the initial stage (no litter) to the latest stage (large quantity of tree litter; $F_{4,30}$ = 2.8, P = 0.006). The ordination was strongly influenced by the enzyme fingerprints of the last stage (large quantity of tree litter), mainly driven by low potential activities of AP, LAP and NAG (Fig. 3). However, removing these samples from the analysis did not notably change the arrangement of the remaining samples in the NMDS ordination (data not shown), indicating that the overall pattern is robust. AP and LAP had the greatest influence on the ordination (r^2 = 0.80 and 0.75, $P < 0.001$), and NAG and BG were also influential (r^2 = 0.31 and 0.24, P = 0.02 and 0.03), as was algal biomass (r^2 = 0.46, $P < 0.001$).

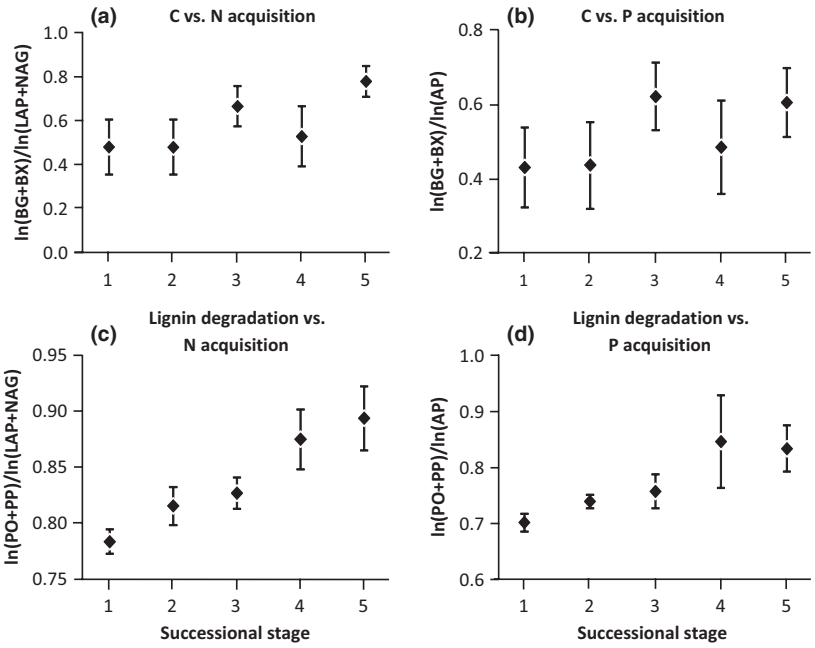

Fig. 2. Ratios of natural log values of potential extracellular enzyme activities in experimental stream channels supplied with different types and amounts of leaf litter: 1) no litter, 2) grass litter, 3) mixed grass and tree litter, 4) tree litter, 5) large quantity of tree litter. Vertical bars denote standard errors calculated across stream channels (n = 3) based on values aggregated within each channel across sampling dates.

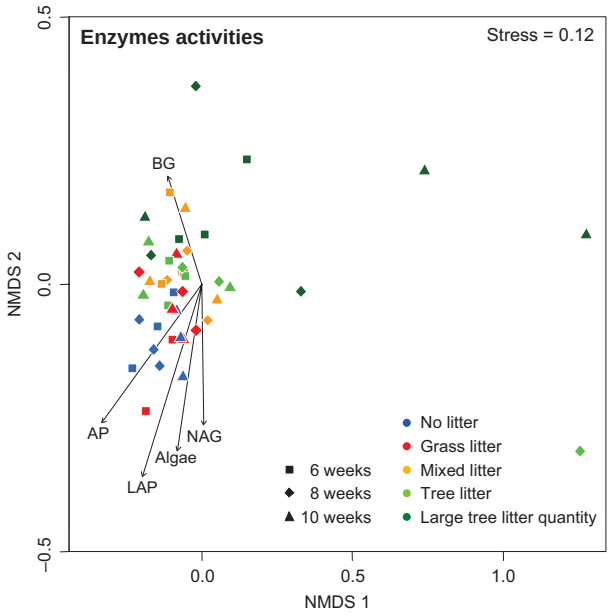

Fig. 3. NMDS ordination of potential extracellular enzyme activity fingerprints in sediments sampled at three occasions in experimental stream channels supplied with different types and amounts of leaf litter. Different symbol shapes denote different sampling dates and colours distinguish different successional stages (i.e. litter treatments). Arrows indicate significant correlations ($P \leq 0.05$) of the ordination with potential extracellular enzyme activities and environmental variables. Algae, algal biomass measured as chlorophyll *a*.

Bacterial and algal biomass

Bacterial biomass in sediment ranged from 0.17 to 0.42 mg C g^{-1} AFDM and was higher in the open-land stages (no litter added or grass litter only) than at later stages with tree litter present (Fig. 4a), although this difference was not significant due to considerable variation among replicate stream channels. No significant differences were found among sampling dates. Algal biomass in sediment ranged from 9.6 to 31.2 mg C g^{-1} AFDM, declining along the experimental gradient from stage 2 onwards (Fig. 4b). This resulted in a significant difference between the two early-successional stages, showing higher algal biomass, and the three later stages (Contrast B: $P = 0.005$), although algal biomass did not differ between successional stages 1 and 3 (Fig. 4b).

Microbial community structure

NMDS analysis of ARISA data, which potentially include chloroplast and mitochondrial DNA, indicate similar patterns when calculated based either on presence/absence data or on relative peak area of OTUs. The analysis revealed similarities of microbial communities in sediments sampled after 6 and 8 weeks (Fig. 5). Samples taken at these times were distinctly separated along the first axis (with two exceptions) from those taken 10 weeks after the start of the experiment ($F_{2,30} = 7.97$, $P = 0.001$). In addition, the microbial communities within both clusters were broadly arranged along a gradient defined by Axis 2, ranging from the initial biofilm stage (no litter) to the late stage with large amounts of tree litter ($F_{4,30} = 3.32$, $P = 0.001$). As a result, microbial communities differed between channels with and without litter (Contrast A: $F_{1,43} = 2.54$, $P = 0.008$), with and without tree litter (Contrast B: $F_{1,43} = 4.15$, $P = 0.002$), with leaves differing in quality

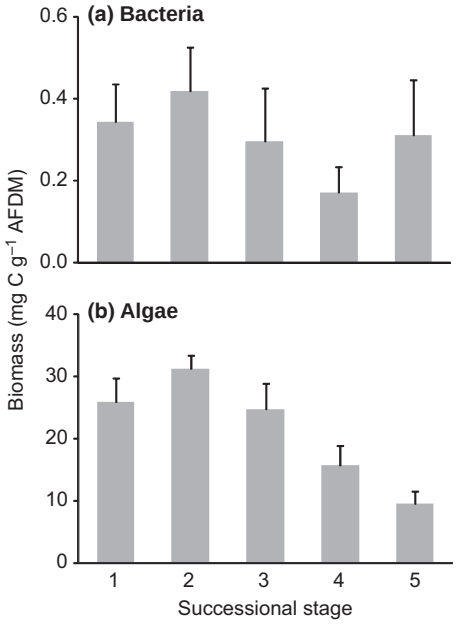

Fig. 4. Bacterial biomass (a) and algal biomass as chlorophyll-*a* content (b) in the sediment of experimental stream channels supplied with different types and amounts of leaf-litter: 1) no litter, 2) grass litter, 3) mixed grass and tree litter, 4) tree litter, 5) large quantity of tree litter. Vertical bars denote standard errors calculated across stream channels ($n = 3$) based on values aggregated within each channel across sampling dates.

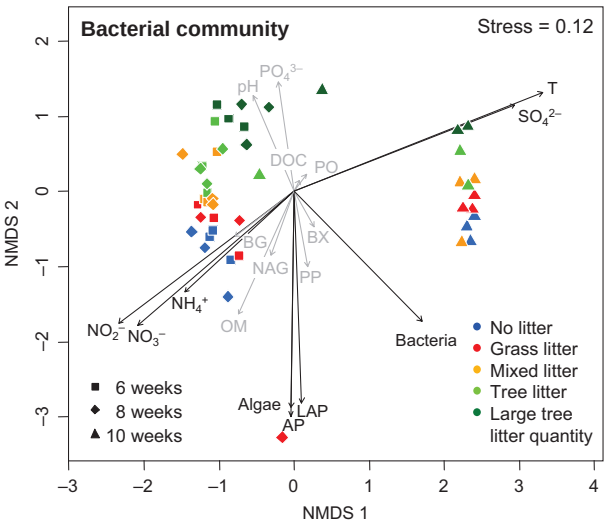

Fig. 5. NMDS ordination of sediment microbial communities sampled at three dates in experimental stream channels supplied with different types and amounts of leaf-litter. Different symbol shapes denote different sampling dates and colours distinguish different successional stages (i.e. litter treatments). Black arrows indicate significant correlations of the ordination ($P \leq 0.05$) with potential extracellular enzyme activities and environmental variables (T = water temperature, Bacteria = bacterial biomass, Algae = algal biomass measured as chlorophyll *a*,). Grey arrows denote variables that were not significantly correlated with the ordination (OM = organic matter content).

(grass vs. tree litter: Contrast C: $F_{1,43} = 2.43$, $P = 0.008$), and with different amounts of tree litter (Contrast D: $F_{1,43} = 2.17$, $P = 0.025$).

Several physicochemical parameters were significantly linked to microbial community structure (Fig. 5). Concentrations of NO_3^-, NO_2^- and NH_4^+ in stream water were correlated with the ordination of the microbial communities in sediments ($r^2 = 0.48$, 0.55 and 0.25; $P = 0.027$ for NH_4^+, else $P < 0.001$), with all arrows pointing in the direction of the early successional stages (no litter and grass litter) and the first two sampling dates (6 and 8 weeks). Water temperature ($r^2 = 0.82$, $P < 0.001$) and SO_4^{2-} concentration ($r^2 = 0.64$, $P < 0.001$) were almost exactly opposed to the N species, pointing towards the last sampling date (10 weeks). Furthermore, algal biomass and potential activities of the nutrient-acquiring enzymes (AP and LAP) were significantly correlated with microbial community structure ($r^2 = 0.51$–0.58; $P < 0.001$), with higher values for all three variables in the early successional stages. Arrows representing these three variables were opposed to pH and PO_4^{3-} (no significant correlation emerging in the ordination) and exactly parallel to Axis 2, just as the gradient defined by the microbial communities. Finally, bacterial biomass was also significantly correlated with microbial community structure ($r^2 = 0.38$, $P < 0.002$), the corresponding arrow pointing towards the early successional stages and the latest sampling date.

Discussion

The results of our experiment mimicking a key aspect of ecosystem succession – leaf litter supply – in outdoor experimental stream channels clearly show that shifts in riparian litter input exert strong effects on microbial community structure and patterns of enzymatic capacities in stream sediments. Changes in microbial communities among successional stages were profound and primarily based on the replacement of certain OTUs, not only on shifts in relative abundances. Specifically, we observed a smooth directional change in microbial communities along the trajectory from channels simulating open-land streams without riparian vegetation to forested streams receiving massive litter inputs. Although the same gradual changes of the communities were apparent 6, 8 and 10 weeks after initiation of the experiment, the communities also clustered according to sampling date, with the shift we observed between weeks 8 and 10 possibly related to the unusually warm weather on the last sampling date (Table 1) or to oxygen depletion increasingly affecting

the sediment microbial communities (L. Gerull *et al.* unpublished data). Nevertheless, these short-term dynamics (i.e. differences between sampling dates) did not mask the influence on the communities caused by our simulated successional stages (i.e. channels receiving litter in different quantities and qualities).

Potential enzymatic activities in sediments can be viewed as indicators of functional microbial responses to environmental conditions such as temperature and particularly nutrient and carbon availability. The patterns shown by several of the measured extracellular enzyme potentials in response to changing litter supply (i.e. at different successional changes), especially those of AP and LAP, are consistent with the observed microbial community changes; both showed a gradual shift across the five litter addition treatments simulating riparian vegetation changes. This finding contrasts with field observations in the study area in real early-successional streams, which were completely devoid of leaf litter. Variation of sediment microbial communities across sites and over time appeared to be completely random in those streams, whereas enzymatic capacities showed pronounced temporal changes (Frossard *et al.*, 2012b). This suggested a decoupling between microbial community structure and ecosystem functioning (Frossard *et al.*, 2012b). Sediment structure, flow, water quality and general environmental conditions of our experimental stream channels and the naturally formed streams in the adjacent catchment were very similar. Thus, the discrepancy in results of both studies lends further support to our conclusion that shifts in litter supply during riparian plant succession can act as a strong structuring force of microbial communities in stream sediments.

In reality, a range of factors other than litter supply are altered during riparian succession but were not specifically considered in our experimental design. These factors include light and temperature regimes, flow conditions, and the supply of DOC and dissolved nutrients (although both shading of the stream bed and P supply by added litter increased along the gradient created by our five litter addition treatments). Together with biological interactions (grazing, competition, facilitation), these environmental factors are likely to superimpose effects of riparian vegetation change to influence stream-bed microbial communities. This notwithstanding, our results reveal that changing litter quality and quantity alone can induce major changes, underlining the power of manipulative experiments to isolate single factors affecting community assembly and function and thus pinpoint a driving force behind the emerging patterns.

Two observations imply that the patterns we observed for various structural and functional variables were influenced more strongly by nutrient acquisition than by carbon demand. The first observation is that trends of the nutrient-acquiring enzymes, but not of the hydrolytic C-acquiring enzymes, were consistent with the overall changes in microbial community structure, as litter supply changed according to the five successional stages simulated in our experiment. LAP, in particular, showed a gradual decrease as litter quantity increased and its quality improved as supplies shifted from grass to tree litter along our experimental gradient. NAG also declined after the two early successional stages devoid of tree litter. Similarly, AP potential activity dropped sharply as soon as litter was available, suggesting that the supply of litter mitigated a P deficiency for microbial growth (although it did not necessarily remove it; see below). This mitigation could be a result of massive leaching of P from dried leaves, which does not generally occur to a similar extent for N (Gessner, 1991) or a reduced demand by algae suffering from light limitation under the litter layer on top of the sediment.

The second indication is that the potential activities of P- and N-acquiring enzymes, AP and LAP, were higher than those of the major carbon-acquiring enzymes measured, resulting in ratios significantly smaller than 1 between the natural log values of potential activities of C-acquiring to P- and N-acquiring enzymes. Analysis of a large set of empirical data yielded an average ratio of 1 : 1 : 1 for the natural log values of potential activities of extracellular enzymes involved in C, N and P acquisition (Sinsabaugh *et al.*, 2009). If this ratio reflects balanced relationships between elemental demand and supply (Sinsabaugh *et al.*, 2009), then one might speculate that the significantly lower ratios we found indicate an imbalance between the elemental stoichiometry of microbial biomass and the available resources. Although speculative, also because freezing of our samples might have resulted in variable effects on different enzymes (Deforest, 2009), and thus to be interpreted with caution, this reasoning would support the idea that the sediment-associated microbial communities in our stream channels could have been nutrient-limited under all litter supply regimes (i.e. at all successional stages).

As extracellular enzyme activities and most other variables were determined in integrated samples over the entire sediment depth of 4 cm and dissolved oxygen was increasingly depleted during our experiment below 0.5–1.5 cm depth (Gerull *et al.*, 2012; A. Frossard & L. Gerull, unpublished data), aerobic microbial metabolism was limited to the upper sediment layers. This could have been a factor accounting for the dramatic shift in microbial community structure observed between weeks 8 and 10 after starting the experiment, although oxygen depletion in sediments did not affect potential activities of hydrolytic enzymes in other studies (Hakulinen *et al.*, 2005; Taylor *et al.*, 2009). Alternatively, the apparent

change in community structure and function could have been influenced by the decreasing importance of algae, whose chloroplast DNA might have contributed significantly to our ARISA community profiles. Therefore, without detailed information on the identity of the DNA fragments, it is not clear to what extent lack of oxygen, changes in other environmental conditions, or shifting importance of algae determined the observed changes in microbial community structure and excretion of microbial extracellular enzymes over the time scale of the present investigation.

The progressive decline in algal biomass – from the simulated open-land stream channels receiving grass litter only to stream channels stocked with tree litter – could have a straightforward reason: reduced average light levels because litter cover increasingly shaded the stream bed. Consequently, the correspondence between algal biomass decline and AP, but not LAP, potential activity could reflect reduced algal phosphorus demand resulting from reduced productivity. Alternatively, the correspondence between declining algal biomass and potential LAP and AP activities could indicate a diminishing algal release of labile organic carbon, reducing the demand of bacteria for nutrient-acquiring enzymes (Jones & Lock, 1993; Espeland *et al.*, 2001), although this relationship has not been clearly described for AP. These two propositions are supported by the higher potential extracellular activities of hydrolytic enzymes measured in autotrophic compared with heterotrophic biofilms (Romaní & Sabater, 2000; Rier *et al.*, 2007; Ylla *et al.*, 2009). Potential activities of C-acquiring hydrolytic enzymes did not decrease with algal biomass, indicating that the influence of algae was either limited or compensated by other factors such as carbon supply by the added litter. Overall, then, both algae and bacteria were likely to contribute to the observed enzyme patterns, although their relative importance might have shifted towards bacteria, as litter inputs provided additional carbon sources and increased shading.

NMDS revealed that changes in microbial communities during ecosystem succession were related to algal biomass and the potential activities of AP and LAP, whereas differences in microbial communities between 6 and 8 vs. 10 weeks after starting the experiment were unrelated to potential extracellular enzyme activities. This suggests a strong link between microbial community structure and ecosystem function along our simulated gradient, but not among sampling dates. This finding contrasts with the correlation between nutrient variables and changes in microbial communities both along the simulated riparian succession and among sampling dates, although the importance of nitrogen and phosphorus changed during the simulated succession but not among sampling dates. Thus, despite the importance of nutrients at the two time scales considered, drivers of long-term successional changes (treatments) and short-term changes (sampling dates) during the experiment were distinctly different overall.

In conclusion, the results presented here from experimental channels mimicking an important aspect of stream ecosystem succession suggests that changes in the quality and quantity of riparian vegetation alone are important enough to induce gradual shifts in microbial community structure, microbial activities related to nutrient cycling, and several other variables describing stream sediments. Evidently, caution is in order when projecting results from a greatly simplified model system to ecosystem succession in real ecosystems, which occurs over decades or hundreds of years and involves numerous additional changes in environmental factors and biological interactions that were not considered in our experimental design. Furthermore, although reflecting natural vegetation changes in our study region, the pattern observed here when a graminoid (*Calamagrostis epigejos*) was replaced by tree litter may not be universal, especially as wood small-reed provides grass litter of unusually low quality (Yuan & Chen, 2009). This notwithstanding, the presented evidence provides a proof of principle and attests that both structural and functional shifts of microbial communities can arise over very short time scales in response to changes in litter supply provided by riparian vegetation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Views of the experimental channels placed adjacent to the experimental Chicken Creek catchment in eastern Germany.